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Chapter 27

ATRAZINE BIODEGRADATION AS RELATED TO THE PHYSIOCHEMICAL PROPERTIES OF A CISNE SOIL FROM A MAJOR ATRAZINE SPILL SITE: ATRAZINE BIODEGRADATION IN A CISNE SOIL EXPOSED TO A MAJOR SPILL

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ABSTRACT

Conventional soil tests, culture-based microbial methods, and the novel method of ^{15}N-DNA stable isotope probing (SIP) were employed to illustrate atrazine biodegradation as related to the physiochemical properties of an atrazine-exposed Cisne soil. This soil exhibited enhanced atrazine degradation. Mineralization underestimated the rate of atrazine dissipation demonstrated by the accumulation of several metabolites. The soil showed high ambient concentrations of NO_3^-; however, NO_3^- did not suppress atrazine degradation. Atrazine natural attenuation was limited by incomplete distribution through the unsaturated soil matrix. Direct plating experiments from the Cisne soil isolated an atrazine-degrading microorganism, ES-1. Analysis of the 16S rRNA gene sequences from the isolate confirmed that ES-1 is closely related (99%) to *Arthrobacter* sp. In pure culture, the isolate rapidly converted atrazine to cyanuric acid. Accumulation of this product was consistent with metabolites in the Cisne soil, suggesting that isolate ES-1 influenced in-situ remediation of atrazine. ^{15}N- SIP experiments were conducted using ^{15}N-*ethylamino*-atrazine. The results of these experiments failed to establish a causal relationship between in-situ atrazine-degradation and ES-1 enrichment; however, these results are likely due to isotopic dilution. Further experiments using ^{13}C-ethyl/isoproylamino-atrazine may yet verify a link between ES-1 and the enhanced natural attenuation exhibited in the Cisne soil.

Keywords: Cisne, Atrazine, stable isotope probing, natural attenuation

1. INTRODUCTION

Atrazine and other s-triazine herbicides have been used for over 50 years for the control of a variety of weeds in agricultural crops, most notably maize (U.S. Department of Agriculture,

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2004). These chemicals are moderately persistent, but also sufficiently water-soluble as to create contamination problems in surface and groundwater (Solomon et al., 1996, Tasli et al., 1996).

Atrazine contamination of water resources is a concern because the compound is a suspected endocrine-disruptor (Hayes et al., 2002, Hayes, 2004). Widespread use of these herbicides has influenced the microbial ecology of agricultural soils world-wide.

The following experiments characterize a Cisne-Darmstadt intergrade (Cisne) soil exposed to high levels of atrazine through a single chemical spill. Large atrazine releases are not uncommon during peak application periods and there is no way to know how many spills go unreported. Therefore, it is important to examine the factors that influence the potential for natural attenuation in soils.

2. MATERIALS AND METHODS

2.1 Chemicals and Materials

Unlabeled atrazine (98%) was purchased from ChemService (West Chester, PA). Uniformly ^{14}C-ring-labeled-atrazine (9.3 mCi per mmol, radiochemical purity ≥95%) was purchased from Sigma-Aldrich (St. Louis, MO). Atrazine-ethylamino-^{15}N (99 atom % ^{15}N) was purchased from Isotec (Miamisburg, OH). Uniformly ^{15}N-ring-labeled atrazine was synthesized from ^{15}N-urea according to the method described in (Bichat et al. 1999). Atrazine metabolite standards: deethylatrazine (2-amino-4-chloro-6-isopropylamino-s-triazine) (98%), deisopropylatrazine (2-amino-4-chloro-6-ehtylamino-s-triazine)(99%), hydroxyatrazine (2-hydroxy-4ethylamino-6-isopropylamino-s-triazine)(99%), deethylhydroxyatrazine (2-hydroxy-4-amino-6-isopropylamino-s-triazine)(98%), and deethyldeisopropylatrazine (2-chloro-4,6-diamino-s-triazine)(99%), were a gift of W. Roy (United States Geological Survey, Champaign, IL). Cyanuric acid (99%) was purchased from Alfa Aesar (Ward Hill, MA). Organic solvents were Optima grade (Fisher Scientific, Pittsburg, PA).

2.2 Enrichment, Isolation, Characterization, and Maintenance of Atrazine-Degrading Microorganisms

Previously researched atrazine-degrading isolates of Pseudomonas sp. Strain ADP and Pseudaminobacter sp. Strain C147 were generously donated by Drs. Larry Wackett and Edward Topp respectively. Atrazine degrading cultures in this study were grown on atrazine mineral salts (AMS) media previously described (Topp et al., 2000a), modified with the addition of 1ml MR2A trace element solution (Atlas, 1997). Post autoclaving, the medium was supplemented with 1ml of a filter-sterilized (0.22 μm, polyethersulfone, Millex-GP,) vitamin solution (Yang and McCarty, 1998) modified with the addition of 0.005 g of thiamine hydrochloride and 0.005 g of nicotinamide L-1, and 1ml of filter sterilized iron stock solution (5g Fe $SO_4 \cdot 7H_2O$ L-1). Solid media preparations consisted of the mineral salts media modified with the substitution of 0.5 g L-1 atrazine instead of 0.025 g L-1 of atrazine (delivered in 1ml of methanol) and supplemented with 15g of Noble agar (Difco, Sparks, MD). Carbon and nitrogen supplied by the vitamin solution were negligible, thus atrazine represented the sole source of those elements.

The concentration of atrazine in the solid media preparations exceeded the solubility limit resulting in a chalky suspension (Mandelbaum et al., 1995). Colonies that developed zones of clearing on the opaque surface of the plates, considered putative atrazine-degraders, were purified by streak plating, and maintained on this medium. Isolates were stored at -80°C in a 15% glycerol solution.

2.3 Properties and Preparation of Soils

The atrazine-contaminated soil, a Cisne-Darmstadt intergrade (Cisne), consisted of surface material excavated five months prior to this investigation from a spill in Patoka, IL. The initial atrazine burden for the Cisne soil is unknown, but exposure was assumed to be significant owing to near complete release of the contents of an applicator truck at the site. The source contamination came from an herbicide tank mix consisting of metolachlor and atrazine. The site had been excavated to a depth of two meters and the contaminated soil was stored under a tarp for approximately six months prior to land application. To obtain soil with the greatest atrazine exposure, the darkest material, presumed to be from the surface horizon, was used for these studies. Reference soils, taken from agricultural production sites throughout Illinois with a known history of atrazine use, were collected from a depth of 0-15cm. These soils included: material from a former pesticide mixing-loading facility (Drummer-chemical loading) and the alfalfa field adjacent to the mixing-loading facility (Drummer-field), two manure-amended agricultural production areas (Thorp and Clarksdale), and the zero nitrogen treatment from the University of Illinois Morrow plots long-term fertility experiment (Flanagan) shown previously to exhibit rapid atrazine mineralization (Sims, 2006). Each of the soils was thoroughly homogenized, sieved to through a 2mm screen, and stored at 4°C. Atrazine histories and taxonomic information for the soils used in this study are described in Table 1. A more detailed account of the Cisne soil properties is listed in Table 2.

Table 1. Physical characteristics, soil taxonomy, and atrazine exposure history for the soils used in this experiment

Soil Name	Abbreviation	Soil Series	Taxonomy	Atrazine History
Cisne	PET	Cisne-Darmstadt intergrade	Fine, smectitic, mesic Mollic Albaqualfs / Fine-silty, mixed, superactive, mesic Aquic Natrudalfs	extensive recent exposure
Drummer-field	GCA	Drummer	Fine-silty, mixed, superactive, mesic Typic Endoaquolls	unknown
Drummer-chemical loading	GCC	Drummer	Fine-silty, mixed, superactive, mesic Typic Endoaquolls	exposure 10years prior
Flanagan	MP	Flanagan	Fine, smectitic, mesic Aquic Argiudolls	continous exposure for >10years
Thorp	CHN18	Thorp	Fine-silty, mixed, superactive, mesic Argiaquic Argialbolls	ammended with manure and exposed to glyphosate and atrazine
Clarksdale	BGWF1	Clarksdale	Fine, smectitic, mesic Udollic Endoaqualfs	ammended with manure and exposed to glyphosate and atrazine

Table 2. Physical and chemical properties of the Cisne soil

Cisne Soil Properties								
%Organic Carbon	Soil pH	Buffer pH	CEC meq/100g	NO3 ppm	NH4 ppm	% Sand	% Silt	% Clay
2.8	6.7	6.9	18.9	161	3	43	44	13

2.4 Determining Atrazine Mineralization Rates of Various Soils

The atrazine mineralization rate for the contaminated Cisne soil was compared to reference soils using laboratory incubations. Owing to the potential for inorganic N sources to suppress atrazine degradation, and a very high residual nitrate concentration in the Cisne soil, a leaching treatment was included to remove excess nitrogen (Mulvaney et al., 2001) from the study soils. Unleached controls were also included for Cisne, Drummer-field, and Drummer-chemical loading. Mineralization of ^{14}C-ring-labeled-atrazine was monitored over a period of 84 days for soils incubated in 473-mL Mason jar microcosms (Mervosh, 1995) at 19°C. Each replicate (three per treatment) received field moist soil (3.5 g on a dry weight basis), which was adjusted to 40% water-filled pore space using an aqueous solution containing uniformly ^{14}C-ring-labeled-atrazine and unlabeled atrazine to deliver a concentration of 30 µg atrazine and 300 Bq ^{14}C / gram soil (dry weight basis). Physical mixing of the treatment solution in the soil was avoided to prevent the collapse of the relatively weak Cisne soil structure and ensure proper aeration at the relatively high water content. Instead, atrazine was allowed to disperse through the soil via advection. Microcosms also contained a 1-mL 0.2M NaOH trap and a 90mm qualitative filter wetted with 0.5 ml buffer (0.575mM KH_2PO_4) to maintain the proper headspace humidity. At 3-7 day intervals the microcosms were aerated and the evolved [^{14}C] CO_2 in the NaOH trap was measured in a 2-mL aliquot using liquid scintillation spectrometry (LSS) in a Packard model 1600TR-Tri-Carb instrument (Packard Instruments, France).

2.5 Soil Incubations with Uniformly ^{15}N/^{14}C Ring-Labeled-Atrazine

Control and treatment samples of the Cisne soil (5 replicates/treatment) were amended twice (Day 0 and 26) with either ^{14}C-ring-UL- atrazine (1110 Bq) and unlabeled atrazine (30µg g-1), or ^{14}C-ring-UL- atrazine (1110 Bq) and uniformly ring labeled 15N atrazine (99 atom%, 30µg g-1). This was accomplished by delivering atrazine to empty scintillation vials in ethyl acetate, which was then allowed to evaporate. Sufficient deionized water was added to dissolve the atrazine and bring the soil to 40% water-filled pore space. Finally, 4 grams (dry weight basis) of the study soil were added to each vial, the vials were placed in sealed microcosms containing a 10-ml 0.1M NaOH trap, and a 90mm qualitative filter wetted with 0.5 ml (0.575 mM) KH_2PO_4 was added to maintain proper humidity. The microcosms were stored at 19°C in the dark. Soils (unlabeled and labeled with 15N-atrazine) were destructively sampled on days: 5, 10, 15, and 41. The procedure described above was used to respike the samples on day 26; however the samples were transferred to new scintillation vials. Between days 15 and 41 the microcosms were opened at 3-4 day intervals and the NaOH trap was sampled for analysis and replaced. The evolved [^{14}C] CO_2 was measured using LSS.

2.6 Atrazine Extraction and HPLC Analysis

On days 5, 10, 15, and 41 soil samples (1 gram dry weight equivalent for each replicate) were removed for DNA extraction using the Powersoil kit (Mobio Laboratories, Carlsbad, CA) according the manufacturer's instructions. The remaining 3 grams of soil were stored at -20°C for chemical analysis. Soil samples (2.5 gram) were transferred to 50–ml Teflon centrifuge tubes and extracting solutions were introduced sequentially to recover atrazine in pools assumed to represent decreasing degrees of microbial bioavailability. Soils were initially extracted with 0.01M $CaCl_2$ (4ml), followed by two methanol extractions (4ml each). Extractions in 0.01M $CaCl_2$ were mixed for 1.5 hours and methanol extractions were mixed for 2 hours on a horizontal shaker and then centrifuged at 17,200g for 15min. Four milliliters of the supernatant were removed from the 0.01M $CaCl_2$ extraction and 1ml was filtered (PTFE, Alltech Associates, Deerfield, IL) prior to reverse phase high performance liquid chromatography (HPLC, Hewlett Packard Series 1050, San Fernando, CA) analyses to measure reversibly-sorbed, bioavailable atrazine. A similar procedure was followed for the first methanol extraction with the exception that only 3.8mls of the supernatant was removed for analysis of the irreversibly-sorbed, potentially bioavailable atrazine. Prior to bound residue analysis, the second methanol extraction was used to remove residual extracting solution (containing atrazine) that remained trapped in the interstitial pore space. This extract was discarded. HPLC conditions were: injector volume, 100µl; mobile phase flow rate, 1.0mL min-1; UV detector wavelength 215 nm; reverse phase C18 column (150mm × 4.6mm, Alltima column, Alltech Associates, Deerfield, IL) and an isocratic mobile phase (methanol:water, 65:35). An apparent Kd value was determined for atrazine and each of the detected metabolites from the ratio of potentially bioavailable (sorbed) to bioavailable (solution) phase material. After the extraction procedure the soil samples were air dried and combusted according to the method described in.Cupples et al. (2000). Finally, to account for any residual radioactivity due to precipitated atrazine, the incubation containers were washed with 1ml of MeOH and the radioactivity in the liquid was measured using LSS.

2.7 Isolation of Atrazine-Degrading Bacteria

Atrazine-degrading bacteria were isolated by selective enrichment. Soil suspensions were directly plated onto semi-selective solid media using the method described in (Weaver et al., 1994). Briefly, 1g soil samples were added to 9.5mls of PBS solution (pH = 8.0) and 0.5g (approximately 15) 3mm autoclaved glass beads in sterile polypropylene 50mL conical tubes (Corning Inc. Corning, NY). Samples were shaken by hand for 1 minute to ensure dispersion of the soil, and then placed on a horizontal shaker for 10min at 160 oscillations per minute. After allowing 30 seconds for the samples to settle, a 1-mL aliquot of the soil suspension from the middle of the tube was used as an innoculum for a dilution series. Samples were diluted in ten-fold serial transfers and 100 µL aliquots from the 10-3 to 10-7 dilutions were spread onto AMS plates. The plates were incubated at 19°C for approximately four weeks. Colonies that developed zones of clearing on the AMS medium were purified by successive streak plating and maintained on the same medium.

2.8 Kinetics of Atrazine Degradation in Pure Culture

The colony producing the largest clearing zone was denoted isolate ES-1 and isolated for further study. A loopful of cells from a purified culture of this isolate was inoculated into culture

flasks containing 2mls LB (Fisher Chemicals) and 3mls AMS. Cells were grown overnight on a rotary shaker at room temperature. The contents of the flasks were pelleted at 3000g and washed once in PBS solution. A single pellet was re-suspended in 40mL liquid AMS supplemented with uniformly ^{14}C ring-labeled-atrazine (650 Bq). The same medium was used for uninoculated controls to confirm the biological basis of atrazine degradation. Atrazine and potential metabolites were determined by reverse phase HPLC using the conditions described earlier. Control and treatment samples were measured after 0, 1, 3, 6, 12, and 24 hours of incubation.

2.9 PCR Amplification of 16S rRNA gene of ES-1

Whole cells from isolate ES-1 and DNA from the Cisne soil were used as template for the amplification of the following genes using the referenced primers: 16S rRNA gene (Liu et al., 1997), atzA, atzB, atzC (Costa et al., 2000), atzD, atzE, atzF (Piutti et al., 2003), trzD (Rousseaux et al., 2001), and trzN (Mulbry et al., 2002). PCR conditions for atrazine-degrading genes were as follows: 94°C (10 min); 94°C, 58°C for atzA, atzD, and trzN / 68°C for atzB / 62°C for atzC, atzD, atzE, atzF, and trzD, 72°C (1 min) (30 cycles); 72°C (10min). PCR conditions for amplifying the 16S rRNA genes were as follows: 94°C (10 min); 94°C (1.5 min), 55°C (1.5 min), 72°C (1.75 min) (25 cycles); 72°C (10min). 25µl PCR reactions were performed according the manufacturer's protocol. PCR products were cloned into Escherichia coli TOP10 using a TOPO TA cloning kit (Invitrogen Corporation, Calsburg, CA). Plasmids were extracted from the cloned cells with a QIAprep miniprep system (Qiagen, Inc., Valencia, CA), and the insertions were sequenced at the W.M. Keck Center for Functional Genomics (Keck Center), UIUC, Urbana, IL.

2.10 Terminal Restriction Fragment (TRF) Profiles

Whole cells from isolate ES-1 were also analyzed to determine its TRF patterns of the 16S rRNA gene patterns obtained after digestion using three restriction endonucleases. The TRF patterns were determined using the standard procedures as outlined in (Liu et al. 1997). PCR primers (Operon Biotechnologies) used were 27F-FAM (5' AGAGTTTGATCMTGGCTCAG, 5' end-labeled with carboxyfluorescein) and 1492R (5' GGTTACCTTGTTACGACTT). PCR mixtures (100µl) included the TaKaRa Ex Taq mixture (Takara Bio), primers (45 pmol each), and 1µl whole cell suspension. The PCR conditions were: 94°C (10 min); 94°C (1.5 min), 55°C (1.5 min), 72°C (1.75 min) (25 cycles); 72°C (10min). PCR products were purified using the QIAquick® PCR purification kit (Qiagen Inc.), according to the manufacturer's instructions. The purified PCR products were separately digested using the restriction endonucleases: HaeIII, RsaI, and MspI according to the recommended protocol (New England Biolabs, Beverly, MA). DNA fragments were separated by capillary electrophoresis (model 3730xl Genetic Analyzer, Applied Biosystems, Foster City, CA) at the Keck Center. The ROX 1000 (Applied Biosystems) internal standard was used to size terminal restriction fragment (TRF) lengths. Data were analyzed with GeneMapper V3.7 software (Applied Biosystems). A separate profile was generated from each sample and restriction endonuclease combination.

2.11 ^{15}N-DNA-SIP of Pure Cultures of Atrazine-Degraders

2.11.1 Bacterial Strains and Culture Conditions

Experiments were performed to determine the feasibility of using ^{15}N-SIP to identify atrazine-degrading organisms in environmental samples. Two isotopically labeled forms of atrazine were used in pure culture experiments with bacterial isolates known to utilize all of the N atoms in the atrazine molecule. Using atrazine-ethylamino-^{15}N as a treatment, two cultures of Pseudaminobacter strain sp. C147 were grown (rotating at 25°C) on AMS media (5mL) supplied with either ^{15}N- or unlabeled atrazine (25mg/L) and then transferred (5% v/v) to media of the same type. To explore the use of uniformly ^{15}N ring-labeled-atrazine as a treatment, cultures of Pseudaminobacter strain sp. C147 and cultures of Pseudomonas strain sp. ADP were grown under the conditions described above on AMS media supplied with either ^{15}N- or unlabeled atrazine. Following growth, cells were harvested from the culture suspension in late exponential growth stage by centrifugation (3000g) and the cell pellets were frozen at -20°C for subsequent DNA extraction.

2.11.2 DNA Extraction and CsCl Density Gradient Ultracentrifugation

DNA from cell pellets was extracted using the DNeasy tissue system (Qiagen, Inc., Valencia, CA) following the manufacturer's instructions for Gram-negative bacteria. DNA was added to a solution of CsCl and Tris-EDTA (TE, pH 8.0). The CsCl/TE starting BD was adjusted to approximately 1.71 g/mL. Ultracentrifugation of samples was performed in Quick-Seal polyallomer tubes (13 X 51 mm, 5.1 ml, Beckman Coulter) in an Optima LE-80K Preparative Ultracentrifuge (Beckman Instruments) outfitted with a VTi 65.2 vertical tube rotor for 48 h, 184 000g (20°C). Buoyant densities (BD) were measured with a model AR200 digital hand-held refractometer (Leica Microsystems Inc. Depew, NY). Following ultracentrifugation, water was injected with a precision pump (model PHD 2000, Harvard Apparatus, Holliston, MA) into the headspace of the centrifuge tube and fractions (75µl) were collected at the bottom as previously described (Cupples et al., 2006, Cupples and Sims, 2007, Lueders et al., 2004). After fractionation, DNA was dialyzed using a 0.025-µm Millipore mixed cellulose ester dialysis filter (Bedford, MA) as previously described (Gallagher et al., 2005). Fractions and purified DNA were stored at -20°C.

2.11.3 Detection of Changes in DNA Buoyant Density

Purified DNA from the atrazine-ethylamino-^{15}N experiment was used as template for PCR amplification of the 16S rRNA gene using the conditions described above. PCR products from labeled and unlabeled incubations were paired according to buoyant density of the template, and separated by electrophoresis on a 1% agarose gel. The effect of the treatment on template buoyant density was determined by comparing the buoyant densities of the heaviest fractions that produced a PCR product. DNA collected in the experiments using uniformly ^{15}N ring-labeled atrazine as a treatment was fluorometrically quantified using the PicoGreen nucleic acid quantification dye (Molecular Probes, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Fluorometry was performed on an Opticon 2 Real Time Thermal Cycler (MJ Research, Bio-Rad Laboratories, Hercules, CA) as previously described (Cupples et al., 2006,

Tian and Edenberg, 2004). The effect of the treatment on template buoyant density was determined by comparing the buoyant densities of the first significant fluorometric peak.

2.12 Environmental ^{15}N-SIP

2.12.1 Incubations with Uniformly ring labeled ^{15}N Atrazine

Incubation and DNA extraction procedures are described earlier. DNA samples taken from the replicates on day 41 were individually separated by ultracentrifugation and purified as described above.

2.12.2 Atrazine-ethylamino-15N incubations

Samples (3 replicates/treatment) were amended once (Day 0) with the following forms of atrazine introduced at 30µg atrazine g-1 soil: unlabeled atrazine (control), atrazine-ethylamino-^{15}N (treatment), or uniformly ring-labeled ^{14}C-atrazine (53 Bq g-1 soil), the latter treatment was included to facilitate radiochemical analysis of atrazine fate. Atrazine solutions were prepared in methanol (3.2-3.5 µg atrazine/µl methanol) and added to the volume of water necessary to bring the water-filled pore space of the soil to 40%. The atrazine/water solutions were deposited onto the bottom of aluminum weigh boats (57mm, Life Science Products, Fredrick, CO) and six grams of the study soil were placed on top of the solution allowing the solution to diffuse through the soil pore space. The weigh boats were placed in sealed microcosms containing a 90mm qualitative filter paper wetted with 0.575mM KH_2PO_4 and a 10ml 0.1M NaOH trap in the radioactive monitoring samples. The microcosms were stored at 19°C in the dark. One gram of soil was removed from each replicate on days 5, 10, 15, 21, 26 and sacrificed for DNA extraction using the Powersoil kit (Mobio Laboratories, Carlsbad, CA) according the manufacturer's instructions. NaOH samples were also collected, as previously described, from the ^{14}C-atrazine treatments on these dates to estimate the percent of compound mineralized in the control and treatment samples. DNA samples from each replicate taken on day 15 were individually separated by ultracentrifugation and purified as described above.

2.12.3 TRF Analysis of Environmental Samples

After fractionation and recovery of DNA, TRF profiles were generated from the 12 heaviest fractions of each sample tube using the primers and PCR conditions described above for the amplification of the 16S rRNA gene. PCR products were purified using the AMPure PCR purification system (Agencourt Bioscience Corp., Beverly, MA) according to the manufacturer's instructions. Purified PCR products were quantified using a UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). Approximately 150ng of the purified PCR products were separately digested using the restriction endonucleases: HaeIII, RsaI, and MspI (New England Biolabs, Ipswich, MA) and the subsequent TRF profiles were generated as described above. Each TRF had a unique fragment length, and a reported peak area of fluorescently labeled product, in arbitrary fluorescence units (FU). Percent abundance of each TRF was determined by dividing the FU under each TRF by the total FU under all the TRFs in the profile as described previously (Abdo et al., 2006, Yu and Chu, 2005). Values given for the percent abundance of

each FU are reported as relative fluorescence units (RFUs). Data sets were constructed of TRFs that were between 50bp and 1000bp in length and greater than 50 FU in height. Each TRF was identified with a sample name, isotopic treatment, buoyant density (determined during fractionation), and RFU value.

2.13 Statistical Analyses

Statistical analyses were developed with the assistance of Charles Smyth, department of Crop Sciences. Analyses were performed using the statistical functions in Excel (Microsoft Corp, Seattle, WA) and SAS (SAS Institute Inc, Cary, NC). The continuous and quantifiable nature of the CsCl density gradient necessitated the use of a covariance model for detecting upward shifts in TRF buoyant density due to isotopic treatment. If the buoyant densities of individual fractions were the same across test tubes then an Analysis of Variance would be appropriate. However, since they are not, it was necessary to include TRF buoyant density as a continuous parameter in the model. An Analysis of Covariance using SAS Proc Mixed was performed on the TRF data generated from the day 15 atrazine-ethylamino-15N incubations and the day 41 uniformly ring labeled 15N atrazine incubations. Analysis was performed using the model:

Yijkl = Isotope i + Test tube (Isotope) j(i) + Densityl + Error k(ijl)

Error = Sample (test tube, isotope, density)

Sample information was entered in the following categories: tube (enzyme), fraction, isotope, density, peak, and RFU. Results were considered significant if the analysis of the effect of isotope produced F values correlating to an α error rate less than 0.25. This error rate is fairly liberal and allows for the detection of TRF buoyant density shifts that may not be due to isotopic treatment; however this liberal error rate allows for the screening and detection of possibly enriched TRFs. TRFs that have been identified as possibly enriched require further analysis using the remaining two enzymes. TRFs with the same relative abundance in TRFLP profiles generated with the remaining enzymes must be examined using the same method. TRFs that comprise similar relative abundances and show similar shifts in buoyant density would be considered enriched. This process could be accelerated through the use of Multivariate Analysis of Covariance to detect concurrent buoyant shifts using all three enzymes simultaneously.

3. RESULTS AND DISCUSSION

3.1 Atrazine Mineralization Kinetics

Atrazine mineralization kinetics for the six soils are given in Figure 1. When compared to five reference soils in our laboratory as well as previous reports in the literature, the Cisne soil appeared to exhibit enhanced atrazine degradation. Leaching did not significantly increase mineralization rates for the Cisne soil, indicating inorganic N concentration was not likely rate limiting. The unleached Cisne soil exhibited the most rapid initial degradation rate (1.822x10-5 mmoles atrazine mineralized day-1) and cumulatively mineralized >82% of the atrazine applied, apparently meeting criteria for enhanced degradation (Zablotowicz et al., 2006). Mineralization

kinetics for the Cisne soil appeared sigmoidal, indicating decreased microbial growth rates with time as local atrazine solution concentration decreased and population numbers increased (Alexander and Scow, 1989). The kinetics of atrazine degradation indicated that degradation was not due to abiotic processes; thus the soil was a good candidate for examination of natural attenuation.

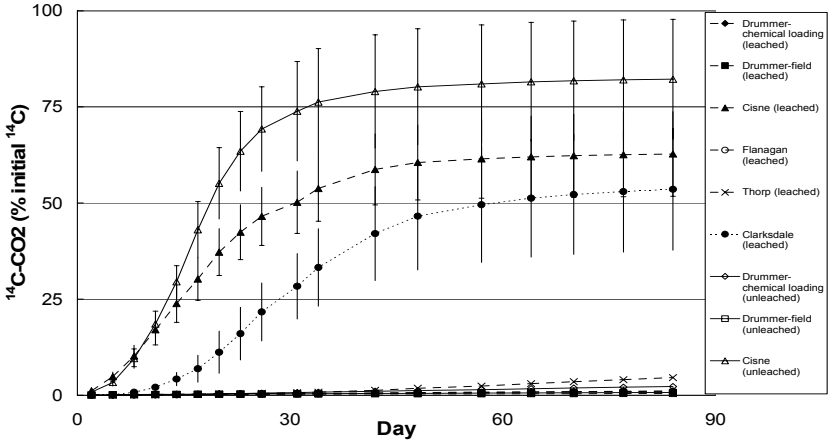

Figure 1. Representative atrazine mineralization in six Illinois soils previously exposed to atrazine. Samples included leached treatments and unleached controls.

To provide a broader context for the Cisne soil findings, mineralization rates were compared to those reported by Zablotowicz et.al (2006) for 7 agricultural soils varying in atrazine exposure history (Figure 2). The Cisne soil exhibited mineralization rates comparable to soils described in the paper as enhanced, likely due to the unusually large exposure to atrazine from the spill. These findings are consistent with atrazine mineralization potential observed for other soils with prior exposure to atrazine (Pussemier et al., 1997, Barriuso and Houot, 1996, Martin-Laurent et al., 2004, Yassir et al., 1999, Vanderheyden et al., 1997). A significant portion of the atrazine applied to the Cisne soil (18%) was not mineralized, and may have been present in the soil as atrazine, metabolites, or bound residues. Incomplete degradation of herbicides has been attributed largely to bioavailability limitations resulting from sorption (Sims and Cupples, 1999) or diffusion limitation through tortuous paths in unsaturated soils (Johnson et al., 1998). Owing to the relatively high carbon content (2.8 %) in the Cisne soil, both of these mechanisms are likely. Mineralization studies were thus followed with more detailed degradation studies in which a mass balance was performed on the C and N added as atrazine to provide a better understanding of material flow in the Cisne soil.

3.2 Soil Incubations with Uniformly $^{15}N/^{14}C$ Ring-Labeled-Atrazine

A mass balance of applied atrazine C and N was obtained by incubating the Cisne soil with uniformly ring-labeled $^{15}N/^{14}C$ atrazine as described in earlier. ^{14}C-mineralization kinetics were calculated as described above, and the distribution of radioactivity among mineralized, bioavailable ($CaCl_2$ extractable), potentially bioavailable (MeOH extractable), and non-extractable fractions was determined at the end of the incubations. After 26 days, the rate of atrazine mineralization in this second incubation was approximately 61% of that observed in the first incubation, likely due to limited bioavailability of solid phase atrazine (Figure 3). Data in

Figure 3 shows no significant label effect on atrazine mineralization. Mineralization had reached a plateau by day 25, thus, though much of the atrazine had not been degraded.

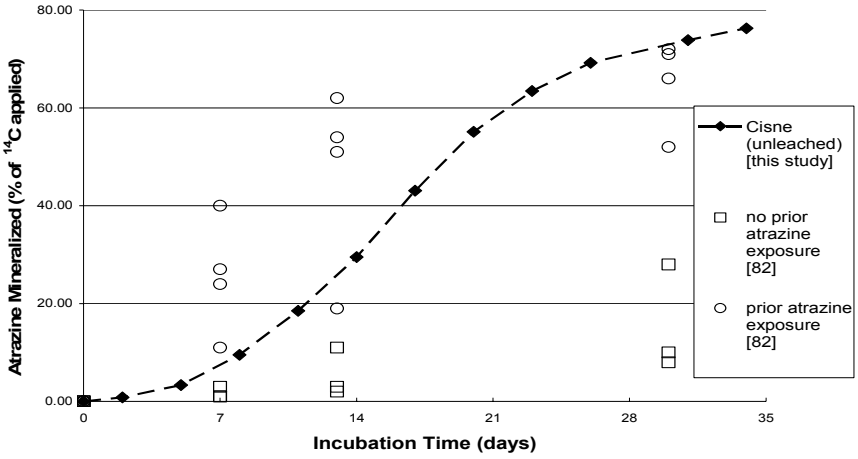

Figure 2. Kinetics of atrazine mineralization in the Cisne soil collected in this study compared to several enhanced (prior exposure) and unenhanced (no prior exposure) soils examined by Zablotowicz et al.

Additional atrazine and water (as described above) were introduced on day 26 (re-spike) to promote degradation. A sharp increase in $^{14}CO_2$ evolution detected on day 26 indicates a response to the re-spike, and suggests that much of the atrazine present at day 25 was no longer bioavailable. The concentration of atrazine used exceeded the solubility limit in this study. Soil moisture content prior to the re-spike was maintained at or below 40% water-filled pore space to ensure proper aeration, and was increased to 50% water-filled pore space during the re-spike process. Thus it is likely that atrazine availability was increased as a consequence of a higher water content and more complete redistribution of the compound through the soil matrix to the active atrazine-degraders. This hypothesis is supported by several observations. Both the bioavailable (aqueous extractable) and potentially bioavailable (methanol extractable) atrazine pools were preferentially depleted to a relatively constant value in the first ten days of incubation with a corresponding release of radiocarbon as CO_2 (Figure 4). A resurgence of mineralization was observed at 41 days (after the 26-day re-spike), even though a considerable amount of atrazine remained present in the system. This increase in mineralization coincided with a decrease in precipitated atrazine, presumably as a result of improved dissipation and redistribution of the chemical due to the additional water added with the re-spike. These findings are consistent with previous work showing increased utilization of an aromatic substrate present in soil solution as water content reached a threshold expected to result in greater continuity of pore space (Johnson et al., 1998). Based on that work, also performed with a Cisne soil, a significant portion of the atrazine present in a bioavailable form would be expected to reside in discontinuous pore space at the lower water content initially used.

When combined, the two extractable fractions accounted for 6.5-18.2% of the initial 14C-atrazine, whereas the non-extractable bound residues only accounted for 2-3% of the initially applied atrazine. The relatively little bound residue detected in the extracted soil is consistent with the unavailability of atrazine ring carbon for incorporation into biomass (Bichat et al., 1999), which would be expected to appear as bound residue in the analysis scheme used here. In a similar study by Houot et al.(2000), soils showing accelerated degradation tended to equally partition residual radioactivity between the extractable and non-extractable fractions, which

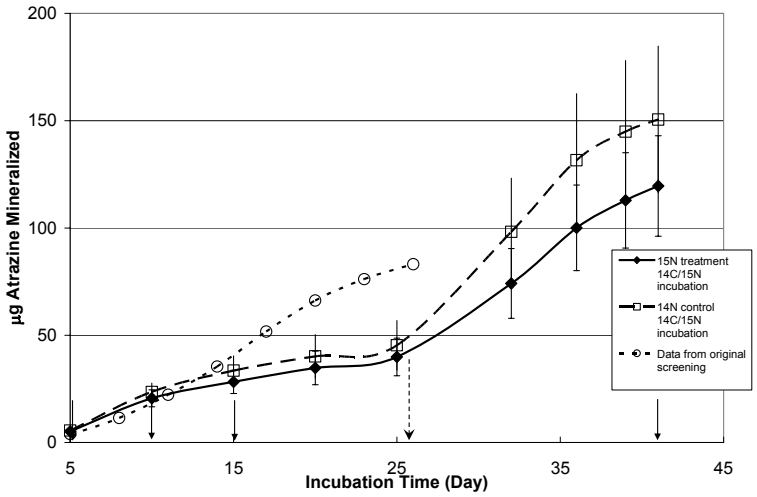

Figure 3. Atrazine mineralization in the Cisne soil incubated with uniformly ring labeled $^{14}C/^{15}N$ atrazine and data from its initial screening . ^{15}N Series includes treatment replicates. Unlabeled series includes control replicates. Solid arrows indicate destructive sampling points for DNA extraction and HPLC analyses. The broken arrow indicates the respike of 30µg atrazine g-1 soil on day 26.

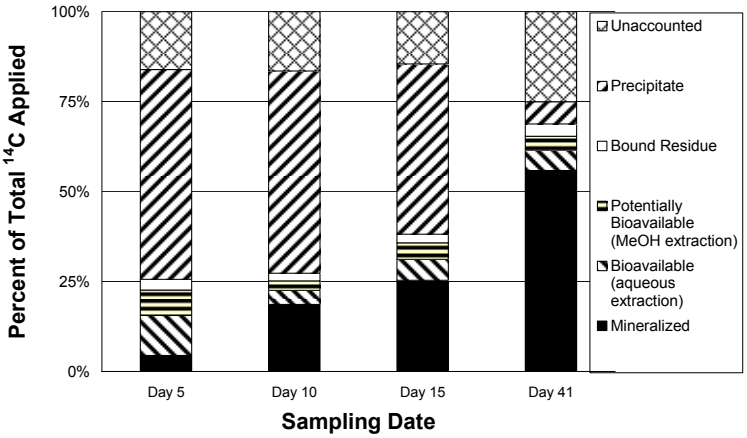

Figure 4. Mass balance of atrazine in the experimental system. Represented as the mean percentage of the total amount of ^{14}C atrazine applied (10 replicates). No distinction is made between control and treatments samples, as the trends were consistent for both groups.

combined to equal 5-10% of the initial atrazine application. The same study showed that in soils showing minimal atrazine degradation, approximately 50% of the initial radioactivity remained extractable and 30% remained as non-extractable bound residues. The fractioning of radioactivity in the Cisne soil more closely resembled the pattern displayed in soils with accelerated degradation than the non-degrading soils described in the Houot study.

Mineralization kinetics in the Cisne soil underestimated the rate of atrazine dissipation demonstrated by the accumulation of several metabolites. In addition to atrazine, three metabolite peaks were detected in the fractions extracted from the Cisne soil. These metabolite peaks corresponded to the retention times for hydroxyatrazine (3.8-4.0min), deethylatrazine and deethylhydroxy atrazine (3.3 min), and the unresolvable peaks of deethyldeisopropylatrazine,

deethyldeisopropylatrazine, and cyanuric acid (2.3-2.5 min), however, these identities have not been confirmed. The metabolites represent approximately 50% of the extracted radioactivity in the bioavailable fraction, but only 20-40% of the extracted radioactivity in the potentially bioavailable fraction (Figure 5), likely owing to the more polar nature of the metabolites.

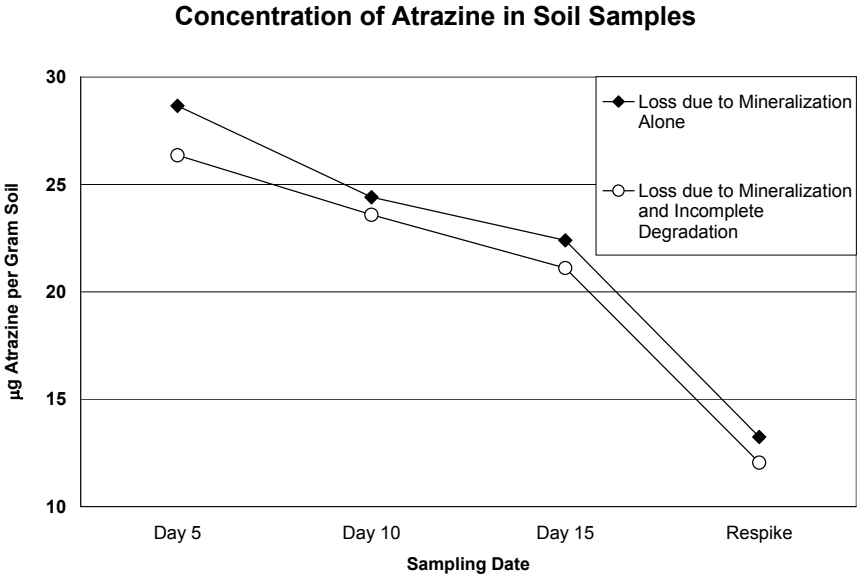

Figure 5. Change in atrazine concentration over time. Mineralization kinetics alone in the Cisne soil underestimate the rate of atrazine degradation .Mean values are presented without standard error bars. Treatment and control replicates exhibited similar results and were grouped together (10 replicates).

3.3 Isolation and Characterization of an Atrazine-Degrading Isolate

The results of metabolite analysis supported the presence of an active population of atrazine-degraders in the Cisne soil comparable to other sites from which such organisms have been successfully isolated (Mandelbaum et al., 1995, Topp et al., 2000a, Topp et al., 2000b Aislabie et al., 2005). Direct soil plating experiments were performed to elucidate some of the microbial interactions taking place in the Cisne soil. Initially, several bacterial colonies showed faint signs of clearing on agar plates that were supersaturated with atrazine. One colony demonstrated distinct removal of particulate atrazine from the medium. This isolate, ES-1, was selected for further study. The atrazine-degrading bacterium formed rounded, shiny white colonies on solid media. Examination of cultures in the exponential growth phase indicated cells were long, slender, Gram-positive rods; however, examination of late-stage cultures indicated cells were Gram-negative cocci.

Analysis of the 16S rRNA gene confirmed the organism to belong the Gram-positive genus Arthrobacter. Isolate ES-1 was identified by comparison of the partial 16S rRNA sequences with bacterial accessions in GenBank using a megablast search (Zhang et al., 2000) and confirming the results using the bl2seq function (Tatusova and Madden, 1999) (open gap penalty=5, extension gap penalty=2). The isolate showed 99% identity with Arthrobacter sp. AD12 (Gen Bank accession AY628690.1) and 97% identity with Arthrobacter aurescens (Gen Bank accession AJ871298) (Table 3).

Table 3. BLAST comparison of sequence identities for 16S rRNA gene and atrazine-degradation genes amplified using isolate ES-1 whole cells as template

Source	Strain	Target Gene	Closest identity in BLAST accession number	Similarity (%)	# Bases used for search	% similarity to corresponding genes encoded by P. ADP catabolic plasmid (U66917.2)
Cisne Soil	ES-1	16S	AY628690.1 16S r RNA from *Arthrobacter* sp. AD12	99	1490	n/a
Cisne Soil	ES-1	*trz*N	AY456696.1 t*rzN* from *Arthrobacter aurescens* strain TC1	99	432	n/a
Cisne Soil	ES-1	*atz* B	AY456696.1 *atzB* from *Arthrobacter aurescens* strain TC1	100	523	100
Cisne Soil	ES-1	*atz* C	AY456696.1 *atzC* from *Arthrobacter aurescens* strain TC1	99	626	99

3.4 Kinetics of Atrazine Degradation in Pure Culture

Pure culture experiments in uniformly ^{14}C ring-labeled-atrazine AMS media were conducted to determine the atrazine-degrading capabilities of ES-1. Mass balance of the culture media indicated no loss of radioactivity, though growth response of the organism was linked to the presence of atrazine in the medium. Analysis of the CO_2 traps suspended in the culture flasks revealed no mineralization, suggesting that the organism degraded atrazine incompletely.

Further time-course kinetic experiments using isolate ES-1 demonstrated rapid dechlorination and dealkylation of atrazine. Results from this kinetic study show that 100% of the atrazine initially added to the culture medium was converted into metabolites. The bulk (>90%) of the atrazine was converted to a polar metabolite with a retention time of approximately 2.3 minutes that co-eluted with authentic cyanuric acid. Approximately 10% of the atrazine in solution was converted to a metabolite that co-eluted with a hydroxyatrazine standard at approximately 4 minutes. The latter compound was detected earlier in the growth medium, suggesting it was a precursor of the terminal product. A number of Gram-positive, atrazine-degrading organisms have been isolated in pure culture including members of the genera: Arthrobacter, Clavibacter, Nocardiodies, and Rhodococcus (Piutti et al.2003, Rousseaux et al., 2001, Topp et al., 2000b, Aislabie et al., 2004, Behki and Khan, 1994, DeSouza et al. 1998, Strong et al., 2002). To date, members of the Arthrobacter genus have demonstrated the most complete degradation of atrazine of all gram positive organisms—metabolizing atrazine to cyanuric acid (Rousseaux et al., 2001, Aislabie et al., 2005, Cai et al., 2003, Strong et al., 2002). These organisms have also shown degradative capacities for a variety of other xenobiotic compounds including pesticides such as: pyridines (O'Loughlin et al., 1999), PCP, phenoxyacetate herbicides, organochlorines, triazones, N-methylcarbonates, N-phenylcarbamates, organophosphates, and glyphosate (De Schrijver and De Mot, 1999).

3.5 Amplification of 16S rRNA Gene Sequence and Atrazine Degrading Genes atzA, atzB, atzC, trzD, and trzN

Isolate ES-1 was identified by comparison of the partial 16S rRNA sequences with bacterial accessions in GenBank using a megablast search (Zhang et al., 2000) and confirming the results using the bl2seq function [105] (open gap penalty=5, extension gap penalty=2). The isolate showed 99% identity with Arthrobacter sp. AD12 (Gen Bank accession AY628690.1) and 97% identity with Arthrobacter aurescens (Gen Bank accession AJ871298) (Table 3).

Genes encoding enzymes involved in atrazine degradation have been characterized including atzABCDEF from Pseudomonas sp. strain ADP (de Souza, M.L., et al. 1998, Piutti et al., 2003, Sadowsky et al., 1998), trzN in Nocardioides strain C190 (Mulbry et al., 2002), and trzD from various gram negative bacteria (Rousseaux et al., 2001). PCR amplification of these atrazine degrading genes in isolate ES-1 using aforementioned primers resulted in an approximately 400bp amplicon for trzN, a 500bp amplicon for atzB, and a 600bp amplicon for atzC. No PCR products were formed using primers specific to atzA or trzD (Table 3). PCR amplification of environmental DNA extracted from the Cisne soil produced amplicons of the expected size for reactions specific to atzABC and trzDN (Table 4); suggesting the presence of other atrazine-degrading organisms besides ES-1. No detectable PCR products resulted from using atzDEF primers when either isolate ES-1 cells or environmental DNA was used as template. Nucleotide sequencing analysis of the cloned genes confirmed that the PCR products were sufficiently homologous to the targeted sequences.

Table 4. BLAST comparison of sequence identities for atrazine-degradation genes amplified using Cisne DNA as template

Source	Strain	Target Gene	Closest identity in BLAST accession number	Similarity (%)	# Bases used for search	% similarity to corresponding genes encoded by P. ADP catabolic plasmid (U66917.2)
Cisne Soil	predominant soil clone	*atzA*	DQ089655.2 *atzA* from *Herbaspirillum* sp. B601 (smzA) gene	99	531	99
Cisne Soil	predominant soil clone	*atz* B	AY456696.1 *atzB* from *Arthrobacter aurescens* strain TC1	100	523	100
Cisne Soil	predominant soil clone	*atz* C	AY456696.1 *atzC* from *Arthrobacter aurescens* strain TC1	99	628	99
Cisne Soil	predominant soil clone	*trz* D	AF086815 from *trzD Acidovorax avenae* subsp. citrulli	100	854	n/a
Cisne Soil	predominant soil clone	*trz* N	AY456696.1 *trzN* from *Arthrobacter aurescens* strain TC1	99	237	n/a

Since the isolate was cultivated from the Cisne soil it is reasonable to expect that atrazine-degrading genes present in ES-1 would comprise a subset of the atrazine-degrading genes residing in the soil metagenome. The presence of putative genes not found in the isolate indicates that other atrazine-degrading organisms are present in the soil. Environmental sequences homologous to atzA support the idea that two competing chlorohydrolases (and populations of atrazine degrading organisms) are present in the same niche environment. The

presence of homologous sequences to the trzD genes indicates organisms other than isolate ES-1 were responsible for the relatively high rate of atrazine mineralization in the Cisne soil. However, microbial degradation of cyanuric acid is relatively common in soil (Cook, 1987, Karns, 1999, Cook et al., 1985) and there may be other cyanuric acid-metabolizing organisms that were not detected with the primers used in this study. The presence of competing chlorohydrolase and aminohydrolase enzymes plus the genetic diversity in the atzBC sequences indicate that multiple and unique atrazine degrading consortia may have been functional in the Cisne soil. Isolate ES-1 was isolated and cultured without enrichment techniques indicating that it may have been one of the more dominant atrazine-degrading microorganisms in the soil, however the genetic diversity exhibited in the soil precludes the possibility that it is the only organism responsible for atrazine degradation.

The detection and isolation of isolate ES-1 helps to explain how atrazine was rapidly degraded in the contaminated soil. The broad substrate range of Arthrobacter species makes them well-suited as agents of bioremediation, especially at sites with multiple contaminants as is often the case with agrochemical contamination. Arthrobacter aurescens strain TC1, also isolated from a highly contaminated soil, has an extremely diverse substrate range and is capable of degrading more s-triazine compounds than any bacterium previously characterized (Strong et al., 2002).

3.6 TRF Analysis of Isolate ES-1

TRFLP analysis of isolate ES-1 pure cultures showed the following TRFs: HaeIII 229bp; MspI 229bp; RsaI 465bp. Each TRFLP profile showed one major peak. These peaks were present in the whole soil TRFLPs, and as noted below, were also detected in soil DNA during the SIP experiments.

3.7 Pure culture ^{15}N-DNA-SIP

DNA based SIP is a relatively new microbial tool used to examine microbial interactions in the environment. Previous research in our lab demonstrated that ^{15}N enriched compounds can be used as substrates for SIP (Cupples et al., 2006, Cupples and Sims, 2007). Experiments were conducted to examine if two different forms of ^{15}N-labeled atrazine, atrazine-ethylamino-^{15}N and uniformly ^{15}N ring-labeled atrazine, could also serve as suitable substrates for SIP-based investigations. Fine fractions were collected and sensitive DNA detection methods were employed to detect small changes in buoyant density. Results from the atrazine-ethylamino-^{15}N experiment indicated that the buoyant density of Pseudaminobacter strain C147 DNA increased from approximately 1.733220 g/ml to 1.736735 g/ml, an increase of 0.003515 g/ml (Figure 6). Results from the uniformly ^{15}N ring-labeled atrazine experiment indicated that the buoyant density increase of Pseudomonas strain ADP DNA was 0.007040 g/ml and the average buoyant density increase of Pseudaminobacter strain C147 DNA was 0.006143 g/ml +/- 0.001663 g/ml. Figure 7 shows how the results of these experiments compare with the buoyant density increases demonstrated in Cupples et al. (2006) and Meselson and Stahl (1958). The buoyant density shifts found in these previous investigations were reported for substrates that were enriched to 100 atom% ^{15}N. The buoyant density increase published in Meselson and Stahl (0.014 g/ml) was used to calculate the theoretical buoyant density increases expected at other enrichment levels. The two N-labeled forms of atrazine used herein were selectively labeled at 100 atom %

^{15}N at the label positions. Incorporation of all five N atoms from atrazine would thus result in enrichment of DNA equivalent to 20 atom % ^{15}N for the ethylamino-labeled atrazine and 60 atom % ^{15}N for the ring labeled material. Based on that assumption, the data from our experiments closely follows the trend projected by the Meselson and Stahl data. Our results demonstrate that addition of substrates with known enrichment levels of ^{15}N will result in reliable increases in DNA buoyant density.

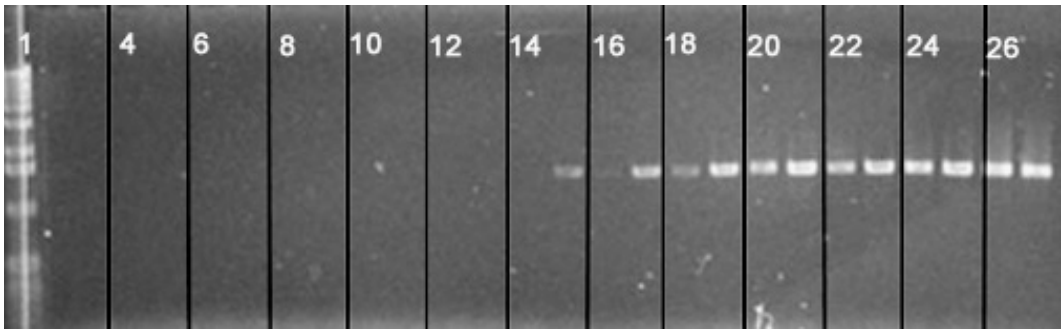

Figure 6. Detection of *Psuedaminobacter* sp. C147 in fractions from a buoyant density gradient using 16S rRNA gene PCR products. Even lanes contain PCR products using template from cells grown on unlabeled atrazine and odd lanes contain PCR products using template from cells grown on ^{15}N-labeled atrazine. Lanes are paired according to closest buoyant density fractions. Lane 1- 1kb ladder. Lane 15- first PCR product detected using ^{15}N enriched *Pseudaminobacter* sp C147 DNA as a template (BD= 1.736735 g/mL), Lane 18- first PCR product detected using , unenriched *Pseudaminobacter* sp C147 DNA as a template (BD = 1.733220).

The consistent relationship between substrate enrichment and increases in DNA buoyant density makes it possible to anticipate the amount of substrate incorporation necessary to result in a detectable increase in buoyant density. In our experiments, the decrease in buoyant density from one fraction to the next is approximately 0.00136 +/- 2.074 E^{-4} g/ml. Using this data, it is possible to extrapolate that at least 13.6% of the nitrogen atoms in the target organism's nucleic acids must be labeled with the ^{15}N isotope in order for the separation of light and heavy DNA to be observed. Nucleic acid enrichment levels below this threshold will not be detectable in our study system.

3.8 ^{15}N-SIP of Environmental Microbial Communities

Most practitioners of stable isotope probing rely on the visible separation of "heavy" and "light" nucleic acids. As our lab demonstrated earlier, enrichment with ^{15}N does not result in a sufficient buoyant density increase to visually resolve enriched and un-enriched nucleic acids from one another (Cupples et al., 2006). In such instances, fine fractionation of the buoyant density gradient must be collected and compared to control samples using quantitative PCR or TRF profiles. Comparison of control and treatment TRFs can be tedious if the effect of the treatment is not pronounced. In such instances, a sensitive detection method is needed. To our knowledge, we have developed the first statistical model capable of distinguishing enriched and un-enriched TRF profiles from one another.

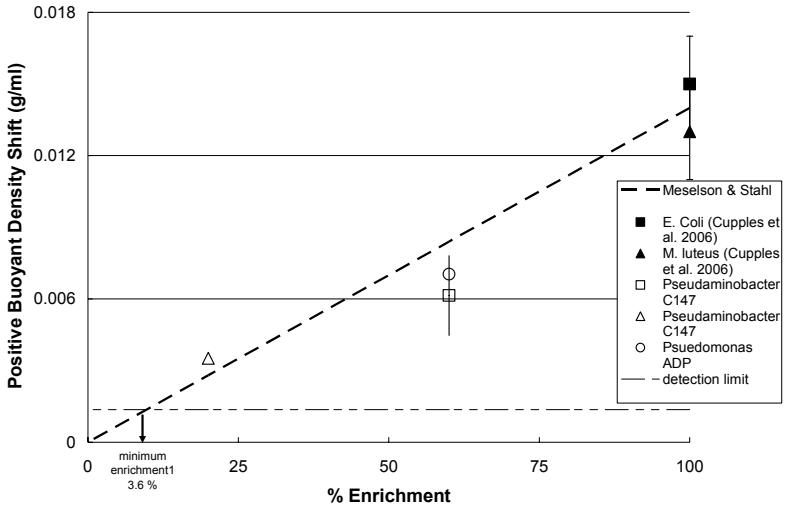

Figure 7. Effect of enrichment with ^{15}N on DNA buoyant density. Closed data symbols show effect of enrichment observed by Cupples et al. [61], and Meselson and Stahl [113]. Open symbols report buoyant density increases demonstrated in the current study. The buoyant density increase published in Meselson and Stahl (0.014 g/ml) for 100% ^{15}N enrichment was used to calculate a theoretical relationship between buoyant density and enrichment indicated by the dashed line.

TRF profiles from soil SIP studies were examined for the three fragments characteristic of isolate ES-1 to determine if the addition of ^{15}N-labeled atrazine resulted in an increase in the organism's DNA buoyant density. As tables 5 and 6 indicate, DNA from isolate ES-1 was not enriched in either the uniformly ring-labeled ^{15}N atrazine incubations or the atrazine-ethylamino-15N incubations

It is expected that treatments of uniformly ring-labeled ^{15}N atrazine would not produce enrichment of DNA in the target organism, since the results of the PCR screening for atrazine degrading genes and kinetic study indicate that isolate ES-1 lacks the genes necessary for ring cleavage. Any incorporation atrazine ring ^{15}N into ES-1 biomass would have been the result of cross-feeding on NH_4^+ (or other products) produced by an organism capable of ring fission. However, this isolate is capable of utilizing the nitrogen present in the alkylamine side chains; therefore, treatments with atrazine-ethylamino- ^{15}N could have produced an effect.

Table 5. Statistical analyses of the TRFLP profiles associated with Isolate ES-1 in the atrazine-ethylamino-^{15}N SIP experiments. Results indicate probability of BD increase due to treatment. Analyses were conducted using 72 observations.

Atrazine-ethylamino-^{15}N SIP							
			mean relative fluorescence of trf				
restriction endo-nuclease	terminal fragment length (trf)	◊ level associated with isotope effect	control	standard error	treatment	standard error	treatment mean signficantly > control
HaeIII	229	0.7575	5.51E-04	3.49E-04	7.19E-04	3.49E-04	no
RsaI	465	0.356	4.60E-05	8.20E-05	1.70E-04	8.10E-05	no
MspI	229	0.5288	4.72E-04	7.00E-05	4.01E-04	7.00E-05	no

Table 6. Statistical analyses of the TRFLP profiles associated with Isolate ES-1 in the uniformly ring-labeled ^{15}N atrazine SIP experiments. Results indicate probability of BD increase due to treatment. Analyses were conducted using 120 observations.

Uniformly ring-labeled ^{15}N atrazine SIP							
			mean relative fluorescence of trf				
restriction endo-nuclease	terminal fragment length (trf)	≅ level associated with isotope effect	control	standard error	treatment	standard error	treatment mean signficantly > control
HaeIII	229	0.3317	6.32E-03	2.68E-03	1.02E-02	2.68E-03	no
RsaI	465	0.1107	2.38E-03	6.79E-04	6.57E-04	6.79E-04	no
MspI	229	0.9543	3.66E-03	2.14E-03	3.49E-03	2.14E-03	no

It is likely that the failure to produce a significant treatment effect in the atrazine-ethylamino-^{15}N incubations is a result of insufficient enrichment of the target nucleic acids. To avoid enrichment-bias atrazine was added to the microcosms at 30µg g-1soil. This is an environmentally relevant concentration of atrazine comparable to the concentrations seen in some agricultural surface soils. As demonstrated in the pure culture SIP experiments, target organisms must utilize the substrate in sufficient quantities to ensure that at least 13.6% of the nitrogen atoms in nucleic acids are ^{15}N -labeled. In pure culture experiments, this parameter is easily controlled; however, in the soil environment there are many competing sources of nitrogen.

The Cisne soil contained excess nitrate either from excess fertilization or mineralization of atrazine N; however, the presence of NO_3- did not suppress atrazine degradation. The study soil contained more than 161 mg/kg NO_3-N; unfertilized Cisne soils typically contain one-tenth this amount of nitrate (Mulvaney et al., 2006). The high nitrate load in the Cisne soil could have competed with atrazine resulting in a dilution of the treatment and a decreased likelihood of detection in SIP. It is possible that the high concentrations of nitrate are the result of the degradation of the initial atrazine and metolochlor spill. The final degradation of atrazine compounds results in the release of NH_4 which in soil is then converted to NO_3^-. Since the soil was stored under a tarp for a number of months before it was remediated the NO_3^- generated from atrazine degradation would not have been leached away. If the high nitrate concentration is due to atrazine degradation, then it is likely that the organisms have a preference for atrazine-N over NO_3^-N, otherwise an accumulation of NO_3^- would not occur. The high nitrate conditions of our study soil create bias for the detection of organisms that prefer atrazine-N over NO_3^-N in the ^{15}N -DNA-SIP experiments.

Despite the high concentrations of NO_3^-N in the study soil it is reasonable to expect some organisms could incorporate significant amounts of ^{15}N into biomass. In the atrazine-ethylamino-^{15}N incubations 180µg of atrazine were added to each sample equaling approximately 12.5µg ^{15}N sample-1. Organisms that only incorporated N from the side chain moieties of atrazine would have accumulated equal amounts of ^{14}N and ^{15}N from the herbicide. The chemical analysis of the Cisne soil (Table 2) indicates that the soil contained 18µg of NH_4^-N and 966µg of NO_3^-N per 6 gram sample. No information is available on the amount of nitrogen contributed by organic matter. If we assume that all identified forms of nitrogen are equally incorporated into biomass then the ratio of ^{15}N:^{14}N is 1:80. However, since the incorporation of NO_3 into biomass requires the input of energy to convert it to NH_4 many microorganisms may prefer less energetically costly forms of nitrogen under aerobic conditions. It is unlikely that

there were substantial anaerobic microenvironments in the atrazine-ethylamino-^{15}N incubations since aggregates greater than 2mm in diameter were removed and research has shown anaerobic microenvironments are unlikely to form in aggregates smaller than 1cm in diameter (Sexstone et al., 1985). Care was taken to avoid disrupting the soil structure and only 40% of the pore space was filled with water. Furthermore, removing excess NO_3 from the Cisne soil through leaching did not show an increase in atrazine mineralization. This may suggest that atrazine-N was preferred over NO_3^-N. Therefore, we will make the assumption that the microorganisms preferentially utilized the atrazine and NH_4^- N before utilizing NO_3^-N for biomass. Under that assumption, the ratio of ^{15}N:^{14}N in the sample is 1:2.4. To achieve a 13.6% ^{15}N enrichment in DNA approximately 1 in 8 N atoms in DNA must be labeled with 15N. The ratio of 1 ^{15}N atom to 2.4 ^{14}N atoms in the readily assimilable nitrogen pool is within the requirements for ^{15}N SIP.

There are several possible reasons why we did not see an increase in the buoyant density of the ES-1 TRFs. Only about 55% of the atrazine added in the ethylamino- ^{15}N incubations was mineralized and metabolite analysis of the uniformly-ring-labeled incubation indicates that approximately 7% of the atrazine applied was incompletely degraded; therefore a conservative estimate would indicate that approximately 62% of the atrazine applied could have been degraded and used for biosynthesis. Using this estimate, the effective ratio of ^{15}N:^{14}N in the sample is reduced to 1:4 assuming only native soil NH_4 competed as an N source for biosynethesis. This ratio is still within the requirement for effective isotopic encorporation, however these estimates assume that all the available ^{15}N was utilized by a single group of atrazine degrading organisms. It is more likely that the ^{15}N label was distributed among several taxa preventing the ES-1 from assimilating enough ^{15}N to significantly increase its BD. Although the ratio of enriched to unenriched readily assimilable nitrogen was favorable, the presence of many atrazine-degrading bacteria would have diluted the treatment effect. This explanation is supported by the presence of atrazine-degrading genes not found in the isolate. If the atzA and trz D genes detected in the Cisne soil code for functional enzymes, then there are several populations of atrazine-degrading bacteria thereby minimizing the incorporation of ^{15}N into the DNA of one organism.

As a member of the Arthrobacter genus, isolate ES-1 probably has a G+C content ranging from 50-70% (Stackebrandt et al., 1983) giving it a relatively high unenriched BD. Our analysis of BD fractions was limited to the 12 heaviest fractions, which included the BD range of ES-1, and favored the detection of organisms with high G+C contents. That isolate ES-1 did not show any enrichment, even though the sampling technique and study design favored its detection, further supports the theory that the effect of the treatment isotope was diluted by multiple atrazine-degrading species and/or incorporation of nitrogen from other sources.

In future experiments these problems can be ameliorated with use of dual-labeled treatments. Experiments by Cupples and coworkers demonstrated that the use of ^{13}C and ^{15}N -dual labeled substrates produced a buoyant density increase of 0.045g/ml when supplied as the sole sources of carbon and nitrogen (2006). A buoyant density increase of that magnitude would simplify detection visually or by comparison of TRFLP profiles. The use of dual-labeled substrates in environmental samples would greatly improve the probability of detection when the treatment is applied at low concentrations.

4. CONCLUSION

Mineralization kinetics appeared to be a conservative estimate of atrazine degradation rates for determining the suitability of this soil for natural attenuation. Natural attenuation of atrazine appeared to be primarily limited by incomplete distribution of the compound through the unsaturated soil matrix, as has been reported for other aromatic compounds under similar conditions. Mineralization kinetics underestimated the rate of atrazine dissipation owing to the accumulation of several metabolites. Additional study soils did not mineralize a significant mass of atrazine, indicating that while natural attenuation was rapid in the Cisne soil, not all soils are suitable for natural attenuation. The Arthrobacter isolate, ES-1, obtained from the Cisne soil without enrichment degraded at an unusually rapid rate, and the termination of the degradative pathway at cyanuric acid was precedented by the atrazine degradative genes detected in the organism. The accumulation of this product was consistent with observations of metabolite accumulation in the soil.

TRFLP analysis revealed fragments consistent with the Arthrobacter sp. in the Cisne soil, though no enrichment of these fragments was observed when the soil was supplied with atrazine labeled with ethylamino- ^{15}N. Results from pure-culture studies with atrazine-degrading organisms indicate that under the right conditions stable-isotope probing SIP may be a useful tool in identifying populations responsible for natural attenuation. The use of fine fractions and statistical analysis coupled with traditional SIP techniques will hopefully allow for the detection of microorganisms responsible for the degradation of nitrogen-containing compounds such as herbicides and explosives. The results of these experiments suggest that this site is a good candidate for remediation by natural attenuation, however stable isotope probing is not a suitable method to elucidate the microbial interactions governing natural attenuation.

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